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Purification and properties of recombinant *Brassica napus* diacylglycerol acyltransferase 1

Kristian Mark P. Caldo [a], Michael S. Greer [a], Guanqun Chen [a], M. Joanne Lemieux [b], Randall J. Weselake [a,*]

[a] Alberta Innovates Phytola Centre, Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada
[b] Membrane Protein Disease Research Group, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada

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ABSTRACT

Diacylglycerol acyltransferase 1 (DGAT1) catalyzes the final step in the acyl-CoA-dependent triacylglycerol biosynthesis. Although the first *DGAT1* gene was identified many years ago and the encoded enzyme catalyzes a key step in lipid biosynthesis, no detailed structure–function information is available on the enzyme due to difficulties associated with its purification. This study describes the purification of recombinant *Brassica napus* DGAT1 (BnaC.DGAT1.a) in active form through solubilization in n-dodecyl-β-D-maltopyranoside, cobalt affinity chromatography, and size-exclusion chromatography. Different BnaC.DGAT1.a oligomers in detergent micelles were resolved during the size-exclusion process. BnaC.DGAT1.a was purified 126-fold over the solubilized fraction and exhibited a specific activity of 26 nmol TAG/min/mg protein. The purified enzyme exhibited substrate preference for α-linolenoyl-CoA > oleoyl-CoA = palmitoyl-CoA > linoleoyl-CoA > stearoyl-CoA.
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1. Introduction

Acyl-CoA:diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) catalyzes the acyl-Coenzyme A (CoA)-dependent acylation of *sn*-1, 2-diacylglycerol to produce triacylglycerol (TAG) and CoA [1]. Two non-homologous endoplasmic reticulum (ER)-bound DGAT families with gene orthologs in a wide range of organisms have been identified, namely DGAT1 and DGAT2 [1]. An ER-bound bifunctional wax ester synthase/DGAT has also been reported in *Arabidopsis thaliana* and it appears to mainly function in wax ester biosynthesis in the stem epidermis [2]. This enzyme is related to the bifunctional wax ester synthase/DGAT previously identified in *Acinetobacter calcoaceticus* [3]. Soluble DGATs have also been identified and characterized in the oleaginous yeast, *Rhodotorula glutinis* [4], peanut (*Arachis hypogaea*) [5,6] and *A. thaliana* [7]. From a biotechnological perspective, *DGAT1* or *DGAT2* has been over expressed in oleaginous plants [8–12] and microorganisms [13,14] including yeast and microalgae, as means of increasing TAG content. In contrast, drugs have been under development for the purpose of inhibiting DGAT activity in humans to combat obesity and related metabolic disorders [1].

The first *DGAT1* gene was identified over 15 years ago [15], and homologous genes have been identified in more than 50 organisms. Despite the importance of DGAT1 in plant, microbial and algal biotechnology, and medicine, there are no reports on the purification of an active DGAT1 to apparent homogeneity from any species. As for many other membrane-bound enzymes, DGAT1 has been recalcitrant to effective purification, presumably due to its hydrophobic properties and low abundance in tissues [1]. Microsomal DGAT activity from germinating soybean (*Glycine max*) was previously solubilized with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), followed by gel filtration, which only produced a partially purified enzyme preparation containing oleosin [16,17]. In addition, DGAT from microspore-derived cultures of *Brassica napus* L. cv Jet Neuf was solubilized using octanoyl-*N*-methylglucamide (MEGA-8) in the presence of 2 M NaCl, and partially purified using gel-filtration chromatography with Sepharose CL-4B as the sieving matrix [18]. The void volume, however, exhibited the highest DGAT activity indicating that the enzyme aggregated during chromatography. One of the four forms of *B. napus* DGAT1 [19], known as BnaA.DGAT1.b (Genbank ID: JN224475), was first cloned by Nykiforuk et al. [20] and the recombinant enzyme produced in *Pichia pastoris* was partially characterized [21]. Recently, recombinant tung tree (*Vernicia fordii*) DGAT1 was produced in *Escherichia coli*, solubilized

Abbreviations: Bna, *Brassica napus*; DGAT, diacylglycerol acyltransferase; DDM, n-dodecyl-β-D-maltopyranoside; DPC, n-dodecylphosphocholine; FPLC, fast protein liquid chromatography; MEGA-8, octanoyl-*N*-methylglucamide; SEC, size-exclusion chromatography; TEV, tobacco etch virus

* Corresponding author. Fax: +1 780 492 6739.
E-mail address: randall.weselake@ualberta.ca (R.J. Weselake).

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with SDS and Triton X-100, and subjected to immobilized nickel ion affinity chromatography [22]. The partially purified DGAT1, however, was reported to be degraded and inactive.

In the current study, recombinant BnaC.DGAT1.a (Genbank ID: JN224473) [19], produced in *Saccharomyces cerevisiae (S. cerevisiae)*, was solubilized with 1% (w/v) n-dodecyl-β-D-maltopyranoside (DDM). Solubilized BnaC.DGAT1.a was then highly purified in active form for the first time using immobilized cobalt ion affinity chromatography followed by gel filtration chromatography. BnaC.DGAT1.a self-associated to form dimers and tetramers, and the purified enzyme effectively utilized a range of acyl-CoAs as fatty acyl donors.

2. Materials and methods

2.1. Construct preparation, expression in yeast and microsome isolation

BnaC.DGAT1.a [19] was cloned into pYES2.1/V5-His TOPO yeast expression vector (Invitrogen), which was subsequently modified using PCR, restriction enzyme digestion and ligation to introduce a tobacco etch virus (TEV) cleavage site before the C-terminal tags and to replace the His6 tag with His10 tag. The primers used for construct modification are listed in Supplementary Table 1. The construct was then used for the transformation of *S. cerevisiae* INVSc1 (Invitrogen) with an S.c. EasyComp™ Transformation Kit (Invitrogen) according to the manufacturer's instruction. Transformed yeast was inoculated into 10 mL 2% (w/v) raffinose synthetic liquid media lacking uracil (0.67% (w/v) yeast nitrogen base and 0.2% (w/v) synthetic complete dropout mix (SC synthetic minimal media)), grown for 24 h at 30 °C with shaking at 220 rpm, and then transferred into 250 mL of the same media for another 24 h. The yeast cells were then used to inoculate SC synthetic minimal media containing 2% (w/v) galactose and 1% (w/v) raffinose at a starting OD600 value of 0.40. Once the OD600 value reached 8, the cells were harvested through centrifugation at 6000×*g* for 10 min. The cell pellets were resuspended in Buffer A (50 mM Tris–HCl buffer (pH 8.0) containing 300 mM NaCl, 10% (v/v) glycerol, 10 mM imidazole, 2 μg/mL pepstatin, 2 μg/mL leupeptin and 2 mM phenylmethylsulfonyl fluoride) and lysed by 2 passes at 35 kpsi through Benchtop homogenizer (Constant Systems). The lysate was spun at 10 000×*g* for 20 min and the supernatant was subjected to ultracentrifugation at 105 000×*g* for 1 h to recover the microsomal fraction. All centrifugation steps were carried out at 4 °C. Protein content was determined using Bradford protein assay (Bio-Rad) with BSA as a standard for most protein samples [23]. For samples containing 1% detergent, Pierce™ BCA Protein Assay Kit was used due to detergent interference with the Bradford protein assay.

2.2. Solubilization of DGAT1

CHAPS, n-lauroylsarcosine, Triton X-100 and Tween-20 were purchased from Sigma. DDM, n-dodecylphosphocholine (DPC), octyl-β-D-glucopyranoside and hexaethyleneglycol monooctyl ether were obtained from Anatrace. MEGA-8 was obtained from Soltec Ventures. BnaC.DGAT1.a in yeast microsomes was solubilized using 1% (w/v) of the above-mentioned detergents in Buffer A at a detergent to protein ratio of 4:1. The mixture was then subjected to ultracentrifugation at 105 000×g for 1 h. The solubilized fractions were incubated with 5× SDS loading buffer at 50 °C for 5 min and resolved using SDS–PAGE followed by blotting onto polyvinylidene fluoride membrane. The recombinant enzyme was detected using anti-V5-HRP conjugated antibody (Invitrogen) coupled with ECL Advance Western Blotting Detection Kit (Amersham) using a variable mode imager (Typhoon Trio+, GE Healthcare). The BnaC.DGAT1.a bands were semi-quantified using ImageJ software [24].

2.3. Purification of DGAT1

Detergent-solubilized BnaC.DGAT1.a was incubated with 3 mL TALON® Metal Affinity Resin (Clontech) for 2 h with rocking at room temperature. After packing the resin into Econo-Column® Chromatography Column, 1.5 × 20 cm (Bio-Rad), it was washed twice with 20 column volumes of wash buffers (Buffer A and Buffer A with additional 5 mM imidazole) and BnaC.DGAT1.a was then eluted in Buffer A, stepwise, with increasing concentrations of imidazole (50–500 mM). SDS–PAGE analysis was conducted to analyze purified fractions. The identity of the band corresponding to BnaC.DGAT1.a was determined through in-gel tryptic digestion coupled with liquid chromatography–tandem mass spectrometry (Institute of Biomolecular Design, University of Alberta). The tag was removed through digestion with TEV protease (Sigma) at an optimized protein-TEV ratio of 0.1 mg of TEV per mg of BnaC.DGAT1.a. Finally, BnaC.DGAT1.a was concentrated using Amicon Ultra-4 centrifugal filter units 50 000 NMWL (EMD Millipore) and loaded onto an FPLC-Superdex 200 13/30 (GE Healthcare Life Sciences) size-exclusion column pre-equilibrated with three column volumes of size-exclusion buffer (25 mM Tris–HCl (pH 8.0) containing 150 mM NaCl, 5% (v/v) glycerol, 1 mM EDTA and 0.05% (w/v) DDM). Size-exclusion standards (Bio-Rad) were used to calibrate the column for the purpose of estimating the apparent molecular mass of the peaks. Protein samples at different stages of purification were analyzed using SDS–PAGE and stained with GelCode® Blue Stain Reagent (Thermo Scientific).

2.4. DGAT1 activity assays

Protein samples at different stages of purification were assayed for DGAT activity in a similar fashion to what was described previously [25]. The enzyme assay was performed at 30 °C for 4 min in a 60-μL reaction mixture containing 333 μM *sn*-1,2-diolein (predispersed in 0.2% (v/v) Tween 20), 15 μM [^{14}C] oleoyl-CoA, 20 mM HEPES-NaOH pH 7.4, 0.46 mM $MgCl_2$ and 0.5–10 μg of protein sample. The enzyme reaction was initiated by addition of the protein sample and quenched with 10 μL of 10% (w/v) SDS. The resulting mixture was applied onto a preparative thin-layer chromatography (TLC) plate (0.25 mm Silica gel, DC-Fertigplatten) with a separate lane for [^{14}C]-triolein standard. The plate was developed with hexane: diethyl ether: acetic acid (80:20:1, v/v/v). The products were visualized by phosphorimaging (Typhoon Trio Variable Mode Imager, GE Healthcare, QC, Canada). Spots corresponding to TAG were scraped and analyzed for radioactivity by Beckman-Coulter LS6500. Under these conditions, no apparent radiolabeled TAG was produced due to the action of endogenous DGAT activity. For the substrate specificity assay, [1-^{14}C]palmitic acid (16:0), [1-^{14}C]stearic acid (18:0), [1-^{14}C]oleic acid (18:1Δ^{9cis}), [1-^{14}C]linoleic acid (18:2$\Delta^{9cis,12cis}$) and [1-^{14}C]α-linolenic acid (18:3$\Delta^{9cis,12cis,15cis}$) were purchased from Perkin Elmer while CoA was obtained from Invitrogen. Thioesters of the abovementioned fatty acids were synthesized as described previously using a bacterial acyl-CoA synthetase (Invitrogen) [26]. Fifteen micromolar of the following [^{14}C] acyl-CoAs were used in the experiment: palmitoyl-CoA (60 Ci/mol), stearoyl-CoA (58.9 Ci/mol), oleoyl-CoA (56.3 Ci/mol), linoleoyl-CoA (58.2 Ci/mol) and α-linolenoyl-CoA (51.7 Ci/mol).

3. Results and discussion

3.1. Production and solubilization of recombinant BnaC.DGAT1.a

Although DGAT1 has already been studied for many years and has been produced in different expression systems, the mechanisms of action and regulation of this enzyme remain unknown.

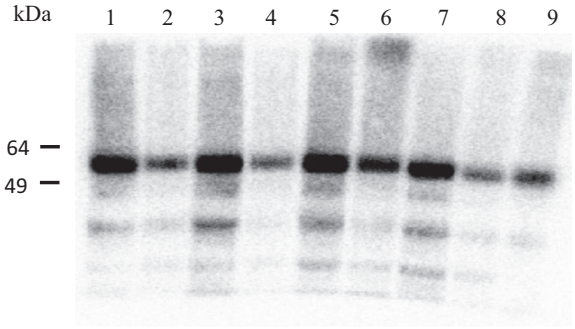

Fig. 1. Western blot profile of solubilized BnaC.DGAT1.a using different detergents. Microsomal proteins (250 μg) were solubilized in 100 μL buffer with different detergents for 2 h at 4 °C, at a detergent concentration of 1% (w/v) and detergent to protein ratio of 4:1. Solubilized enzyme recovered in supernatants obtained following centrifugation at 105 000×*g* for 1 h was resolved by SDS–PAGE, blotted onto polyvinylidene fluoride membrane and detected using anti-V5-HRP antibody coupled with enhanced chemiluminescence detection system. Ten μL of supernatant were applied per lane of the SDS gel. Detergents used: (1) n-lauroylsarcosine; (2) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; (3) n-dodecylphosphochline; (4) Tween-20; (5) Triton X-100; (6) hexaethyleneglycol monooctyl ether; (7) n-dodecyl-β-D-maltopyranoside; (8) octyl-β-D-glucoside; (9) octanoyl-*N*-methylglucamide.

Detailed structure–function analysis requires purified proteins and previous purification studies on DGAT1 had met with limited success. *In silico* topology analysis of DGAT1 sequences showed that the enzyme appears to contain 8–10 transmembrane segments [1], while experimental analysis of murine (*Mus musculus*) DGAT1 using protease protection assays and indirect fluorescence showed that the enzyme has three transmembrane regions [29]. The presence of transmembrane domains poses a challenge to DGAT1 purification since the enzyme must be first solubilized with detergents that maintain enzyme activity.

In the current study, *BnaC.DGAT1.a* was expressed in *S. cerevisiae* using a modified pYES2.1 vector to produce a microsomal fusion protein having a C-terminal V5 epitope-His10 tag linked by a TEV cleavage site. Nine detergents were then tested for their ability to solubilize the microsomal enzyme (Fig. 1). The expressed protein was detected through Western blotting using antibodies against the V5 epitope. BnaC.DGAT1.a was maximally solubilized with n-lauroylsarcosine, Triton X-100, DDM and DPC; all of which solubilized more than 80% of the total enzyme polypeptide. The remaining detergents evaluated solubilized less than 50% of the microsomal enzyme. No apparent formation of SDS-resistant multimer of BnaC.DGAT1.a was detected following analysis of the solubilized fractions. Less than 10% of total enzyme activity was recovered using n-lauroylsarcosine, Triton X-100, DDM or DPC. Purification of DGAT1 was then pursued using DPC and DDM due to their non-denaturing properties and compatibility for structural studies.

3.2. Purification and molecular mass analysis of BnaC.DGAT1.a

Initial attempts at purification showed that BnaC.DGAT1.a was less prone to aggregation and inactivation when solubilized in DDM than in DPC. Therefore, large-scale purification studies used DDM for solubilization of the enzyme. Preliminary optimization of DDM concentration showed that higher total enzyme activity can be recovered using a detergent concentration less than 0.5% (w/v) in the presence of 2 M NaCl. The high concentration of NaCl, however, interfered with cobalt affinity chromatography. Higher yield could still be obtained after immobilized metal ion affinity chromatography when the recombinant enzyme was initially solubilized with 1% (w/v) DDM in the presence of 0.3 M NaCl (as in Fig. 1, lane 7). Although known to be a mild detergent, DGAT activity dropped by 16-fold following solubilization with 1% (w/v)

Table 1
Purification of BnaC.DGAT1.a from 3-L yeast culture.

Fractions	Volume (ml)	Total activity (nmol TAG/min)	Total protein (mg)	Recovery (%)	Specific activity (nmol TAG/min/mg protein)	Purification fold
Microsome	60.0	787.46	231.24	100	3.41	1.0
Solubilized fraction	58.0	33.64	162.89	4.3 (100)[a]	0.21	0.06 (1.0)[a]
ICAC	4.0	45.97	2.26	5.8 (137)[a]	20.39	6.0 (98)[a]
Peak II of SEC	1.0	1.68	0.065	0.2 (5)[a]	26.00	7.6 (126)[a]

[a] Recovery (%) and purification fold relative to the solubilized fraction. ICAC, immobilized cobalt ion affinity chromatography; SEC, size-exclusion chromatography; TAG, triacylglycerol.

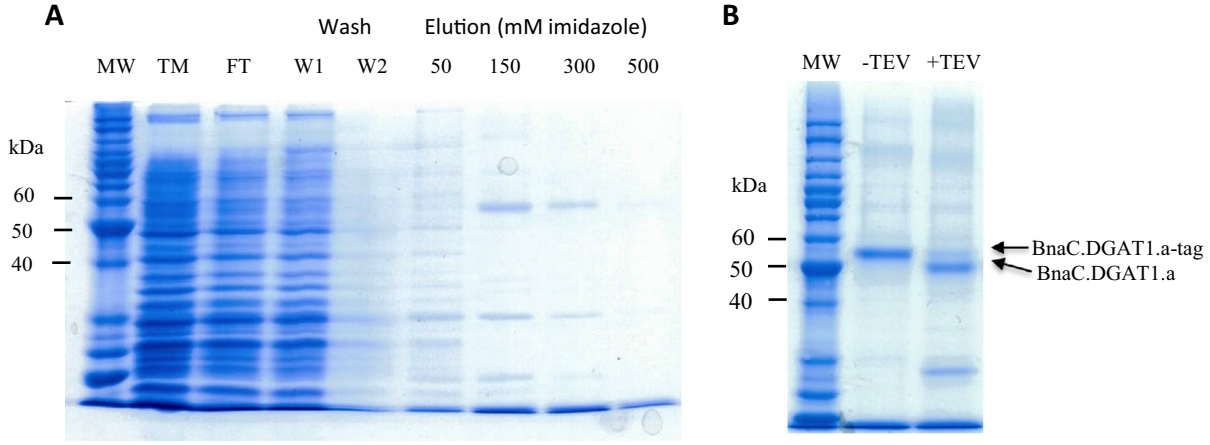

Fig. 2. Partial purification of BnaC.DGAT1.a. by immobilized cobalt ion affinity chromatography followed by removal of the C-terminal tag. (A) SDS–PAGE of column fractions. TM, total microsomal proteins; FT, flow-through. Ten μL of TM, FT, Wash 1 and Wash 2 were loaded while 20 μL of eluents were used. (B) SDS–PAGE of pooled fractions (elution with 150 mM and 300 mM imidazole) before and after treatment with tobacco etch virus (TEV). Twenty μL of sample were loaded per lane.

Table 2
Unique peptides identified for BnaC.DGAT1.a derived from in gel trypsin digestion coupled with analysis by LC–MS/MS.

Sequences	
ANLAGENEIRESGGEAGGNVDVR	ANPEVSYYVSLK
SDSSnGLLPDSVTVSDADVR[a]	GDLLYGVER
VRESPLSSDAIFK	ANLAGENEIR
SDSSNGLLPDSVTVSDADVR	ESPLSSDAIFK
TLANSSDKANPEVSYYVSLK	

[a] n corresponds to deaminated asparagine.

DDM (Table 1). The reduced enzyme activity may be attributed to the high aggregation number of DDM and the low stability of the active DGAT1 conformation in detergent micelles. DDM molecules may have prevented the entry of hydrophobic substrates into the active site of the BnaC.DGAT1.a. It can be noted, however, that the specific activity of BnaC.DGAT1.a increased as purification progressed.

DDM-solubilized BnaC.DGAT1.a was partially purified using immobilized cobalt ion affinity chromatography. A His10 tag at the C-terminus was found to be effective for immobilized metal ion affinity chromatography after finding that the His6 tag was not effective in binding the recombinant enzyme efficiently in the presence of detergent. The eluted fractions were analyzed using SDS–PAGE, and BnaC.DGAT1.a was effectively eluted from the resin, stepwise, over a range of 150–300 mM imidazole (Fig. 2). The band corresponding to BnaC.DGAT1.a had an apparent molecular mass of 58 kDa, which is slightly smaller than the theoretical mass of 64 kDa. This phenomenon has also been observed for other membrane proteins [27]. In gel digestion coupled with LC–MS/MS analysis verified the identity of the target polypeptide as BnaC.DGAT1.a with the unique fragments identified in Table 2. The expression yield post-purification with cobalt ion affinity resin was 0.75 mg per liter of yeast culture.

The partially purified enzyme was subjected to TEV protease digestion to remove the purification tag. The enzyme solution was then concentrated and subjected to size-exclusion chromatography (SEC) to further purify the enzyme (Fig. 3A). Analysis of SEC fractions by SDS–PAGE indicated that BnaC.DGAT1.a could be resolved into different oligomerization states (Fig. 3A and B). BnaC.DGAT1.a polypeptide was present in the void volume and

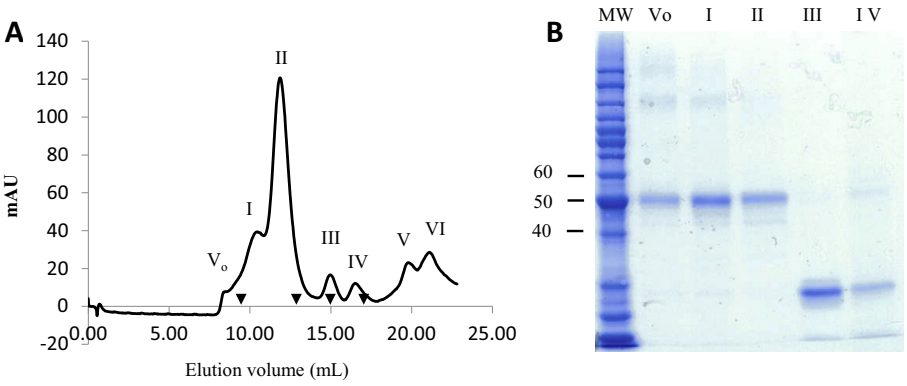

Fig. 3. Purification of BnaC.DGAT1.a by FPLC size-exclusion chromatography using a Superdex 200 10/30 column. (A) Elution profile of proteins resolved by gel-filtration chromatography. The column calibration standards: bovine thyroglobulin (670 kDa, Stokes radius 86 Å), bovine γ-globulin (158 kDa, Stokes radius 51 Å), chicken ovalbumin (44 kDa, Stokes radius 28 Å), and horse myoglobin (17 kDa, Stokes radius 19 Å) were eluted at 9.73, 12.73, 15.26 and 17.06 mL (indicated by triangles), respectively. (B) SDS–PAGE of fractions from size-exclusion chromatography. All peaks except peak II were concentrated five times prior to SDS–PAGE and 20 μL of each peak was loaded in the gel. This corresponds to 0.53, 2 and 1.28 μg protein for Vo, I and II, respectively.

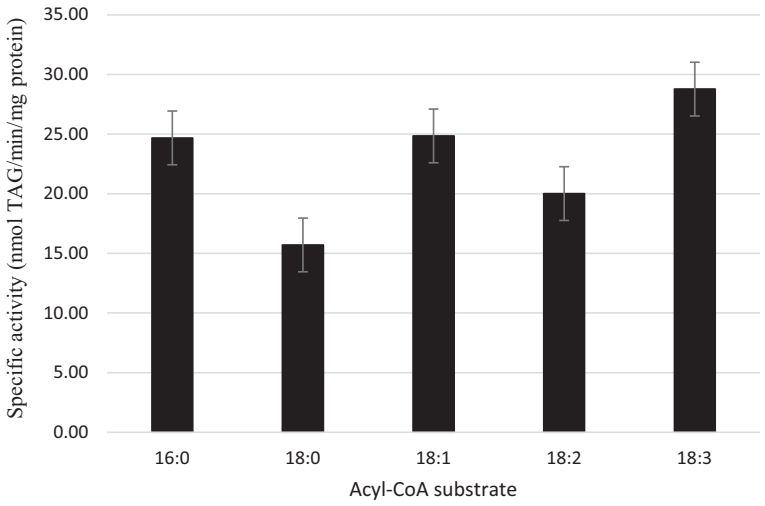

Fig. 4. Acyl-CoA substrate specificity of purified BnaC.DGAT1.a in n-dodecyl-β-D-maltopyranoside micelles. Three hundred-thirty nanograms of protein were used per reaction mixture with an acyl-CoA concentration of 15 μM. Enzyme reactions were allowed to proceed at 30 °C for 4 min prior to quenching with SDS. All reactions were performed in triplicate and error bars correspond to +/− 1 standard deviation. Radiolabeled acyl-CoAs used: palmitoyl (16:0)-CoA; stearoyl (18:0)-CoA; oleoyl (18:1)-CoA; linoleoyl (18:2)-CoA; α-linolenoyl (18:3)-CoA.

in two additional peaks that were resolved within the sieving range of the column. Regression analysis using a standard curve revealed that the major peak containing BnaC.DGAT1.a exhibited an apparent molecular mass of 214 kDa. If one DDM micelle with roughly a molecular mass of 45 kDa is associated with one BnaC.DGAT1.a (with a molecular mass of 58.5 kDa after TEV cleavage), one BnaC.DGAT1.a-DDM micelle has a total molecular mass of about 103.5 kDa [28]. It can then be inferred that the major peak is composed of a dimeric enzyme. Another peak containing BnaC.DGAT1.a, was eluted earlier and was roughly one-third the height of the major peak. This peak has an apparent molecular mass of 414 kDa, which is almost twice the size as the major peak, suggesting that the enzyme can also form tetramers. The current observations on self-association of BnaC.DGAT1.a are consistent with previous crosslinking studies with a recombinant hydrophilic N-terminal fragment of BnaA.DGAT1.b and murine DGAT1, which were shown to form dimers and tetramers [29,30]. In addition, McFie and colleagues [29] showed that murine DGAT1 lacking residues 2–84 can still form dimers but not tetramers and this truncated form has ~14-fold increased activity over the full-length enzyme. The oligomerization of DGAT1 may be an important phenomenon related to the mode of enzyme regulation that will need further investigation. In the current study, the symmetry of the prominent second peak in the size-exclusion profile (Fig. 3A) suggested that this enzyme fraction was suitable for use in crystallization trials. The specific activity of BnaC.DGAT1.a in peak II was 26.0 nmol TAG/min/mg protein (Table 1) using [1-^{14}C]oleoyl-CoA and *sn*-1,2-diolein as substrates. Overall, the enzyme was purified about 7.6-fold over the microsomal fraction and 126-fold over the solubilized fraction (Table 1). The purified enzyme could be stored at 4 °C for a week without any significant loss in activity.

3.3. Acyl-CoA substrate specificity of purified BnaC.DGAT1.a

Acyl-CoA substrate specificity assays of purified BnaC.DGAT1.a, conducted using 15 μM acyl-CoA, indicated that the enzyme could effectively utilize a range of different substrates (Fig. 4), which represented the fatty acids typically found in the seed oil of *B. napus* DH12075 [31]. BnaC.DGAT1.a exhibited the highest activity with α-linolenoyl-CoA. Similar enzyme activities were obtained when oleoyl-CoA or palmitoyl-CoA were used as acyl donors, which were 85–86% of that obtained using α-linolenoyl-CoA. The enzyme exhibited the least preference for linoleoyl-CoA and stearoyl-CoA, which were 70% and 54% of the activity, respectively, with α-linolenoyl-CoA. These results are in general agreement with previous acyl-CoA specificity assays of a MEGA-8-solubilized DGAT activity from microsomes of microspore-derived embryos of *B. napus* L. cv Reston where the solubilized enzyme showed a greater preference for palmitoyl-CoA or oleoyl-CoA over stearoyl-CoA at substrate concentrations ranging from 5 to 20 μM [18]. The increased specificity for α-linolenoyl-CoA observed in the current study is interesting in the light of previous findings where antisense suppression of *BnaDGAT1* was shown to decrease the proportion of α-linolenic acid in the seed oil of transgenic *B. napus* DH12075 [31].

4. Conclusions

In summary, this study reports the first successful purification of a DGAT1 in active form. The selection of a suitable detergent was found to be crucial in purification along with the size of the poly-His tag used to facilitate immobilized cobalt ion affinity chromatography. Although solubilization with DDM reduced DGAT1 activity, the activity was recovered after column chromatography. The results of SEC confirmed previous findings that the enzyme exists in different multimeric forms. Lastly, the substrate specificity study indicated that BnaC.DGAT1.a could use a range of different acyl-CoAs, with the most effective substrate being α-linolenoyl-CoA. Overall, the successful purification of BnaC.DGAT1.a in active form sets the foundation for the in-depth evaluation of structure–function in this biotechnologically important enzyme.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.02.008.

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